INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 93/02604

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07D498/18 C07F7/18 A61K31/435 //C07D498/18,311:00, 273:00,221:00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07D C07F A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,5 151 413 (C. E. CAUFIELD ET AL) 29 September 1992 see claims 1,13	1,7
X	US,A,5 120 842 (A. A. FAILLI ET AL) 9 June 1992 see claim 1	1

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
14 December 1993	28. 12. 93

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Voyiazoglou, D

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 93/02604

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-5151413	29-09-92	NONE		
US-A-5120842	09-06-92	AU-A-	1389392	08-10-92
		EP-A-	0507556	07-10-92
		JP-A-	5078377	30-03-93

Annu. Rev. Immunol. 1996. 14:483–510

IMMUNOPHARMACOLOGY OF RAPAMYCIN[1]

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KEY WORDS: immunosuppression, cell cycle, protein synthesis inhibitor, lymphocyte activation, growth control

ABSTRACT

The potent immunosuppressive drugs FK506 and rapamycin interfere with signal transduction pathways required for T cell activation and growth. The distinct inhibitory effects of these drugs on the T cell activation program are mediated through the formation of pharmacologically active complexes with members of a family of intracellular receptors termed the FK506 binding proteins (FKBPs). The FKBP12 · FK506 complex specifically binds to and inhibits calcineurin, a signaling protein required for transcriptional activation of the interleukin (IL)-2 gene in response to T cell antigen receptor engagement. The FKBP12 · rapamycin complex interacts with a recently defined target protein termed the mammalian target of rapamycin (mTOR). Accumulating data suggest that mTOR functions in a previously unrecognized signal transduction pathway required for the progression of IL-2-stimulated T cells from G_1 into the S phase of the cell cycle. Here

[1]Abbreviations: AT, ataxia telangiectasia; CaN, calcineurin; cdk, cyclin dependent kinase; CLN, cyclin; CRC, calcium release channel; CsA, cyclosporin A; eIF, eukaryotic initiation factor; FKBP, FK506 binding protein; IFN, interferon; IL, interleukin; IP3R, inositol trisphosphate receptor; Kip, cyclin-dependent kinase inhibitor protein; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T cells; ORF, open reading frame; PHAS, phosphorylated heat-and-acid stable; PI 3-kinase, phosphatidylinositol 3-kinase; PI 4-kinase, phosphatidylinositol 4-kinase; PPIase, peptidyl-prolyl isomerase; RAP, rapamycin; RyR, ryanodine receptor; TcR, T cell receptor; TOR, target of rapamycin

0732-0582/96/0410-0483/$08.00

we review the immunopharmacology of rapamycin, with particular emphasis on the characterization of mTOR.

INTRODUCTION

The generation of immune responses to most antigenic stimuli is crucially dependent on an initial phase of T cell activation and proliferation. Activation of T cells is conventionally divided into two sequential stages. Prior to recognition of its cognate antigen, the mature T cell resides in the G_0 phase of the cell cycle. During the first stage of activation, contact with an antigen-presenting cell delivers the signals required for cell-cycle entry (G_0- to G_1-phase transition) and the expression of high-affinity receptors for T cell growth factors, including interleukin (IL)-2 and IL-4. The initial activation step also elicits the production of IL-2 and other growth-promoting cytokines by a subset of T cells within the activated population. The subsequent binding of T cell growth factors to their high-affinity receptors initiates the signaling events required for the progression of the G_1-phase T cell into S phase and, ultimately, into mitosis.

The bacterially derived immunosuppressants FK506 (tacrolimus, Prograf) and rapamycin (sirolimus, Rapamune) have proven to be powerful pharmacologic probes for the dissection of signal transduction events related to T cell activation and growth. Although both drugs bind to the same immunophilin receptor, the resulting immunophilin · drug complexes interfere with distinct intracellular signaling pathways in T and other types of cells. Treatment of T cells with FK506 specifically inhibits the activity of the Ca^{2+}-regulated serine-threonine phosphatase, calcineurin, an enzyme required for the transmission of T cell activating signals from the T cell antigen receptor (TcR). In contrast, the formation of immunophilin · RAP complexes in T cells interferes with the abilities of T cell growth factors to drive the progression of these cells from G_1 to S phase of the cell cycle. Recent studies have identified homologous target proteins for the immunophilin · RAP complex in yeast and mammalian cells. Ongoing studies with RAP as a probe are beginning to uncover a novel signal transduction cascade that may play a general role in the regulation of cell cycle progression in lymphoid and other eukaryotic cells.

DISCOVERY AND IN VIVO IMMUNOSUPPRESSION

Rapamycin (RAP), a lipophilic macrolide, was identified more than twenty years ago during antibiotic screening at Ayerst Research Laboratories. Produced by a strain of *Streptomyces hygroscopicus* isolated from a soil sample

obtained from the Vai Atore region of Easter Island (Rapa Nui) (1), RAP is a white crystalline solid (m.p. 183–185°C), virtually insoluble in water but readily soluble in ethanol, methanol, dimethylsulfoxide, and other organic solvents (2). Although lacking antibacterial activity, RAP is a potent inhibitor of yeast growth and a moderate growth inhibitor of filamentous fungi (2). It is most active against species of *Candida*, particularly *C. albicans*, and protects against systemic and vaginal candidosis in mice, without acute toxicity (LD_{50}: 597 mg/kg intraperitoneally) (3). Early mechanistic studies showed that concentrations of RAP as high as 1.0 μg/ml do not inhibit *C. albicans* growth during the first hour after addition to the growth media, but that concentrations as low as 5 ng/ml are growth-inhibiting after 90 min (4). In addition to fungicidal activity, RAP exerts tumoricidal activity (5), establishing that the antiproliferative effects observed in yeast can be extended to mammals. Labeling studies in *C. albicans* demonstrated that RAP strongly inhibits the incorporation of [^{32}P] phosphate into DNA and RNA (4), an indication that the drug exerts an effect upon the cell cycle. The structural characterization of RAP ($C_{51}H_{79}NO_{13}$; Figure 1) showed the molecule to be a mixture of two conformational isomers due to *cis-trans* rotation about an amidic bond in the 31-membered macrolide ring (6). The proposed chemical structure (6) was confirmed by the total organic synthesis of RAP (7–10).

The first demonstration of RAP's immunosuppressive activity was obtained from studies showing its inhibitory effects upon production of humoral IgE as well as its preventative effects in two animal models of human autoimmune disease, experimental autoimmune encephalitis and adjuvant arthritis

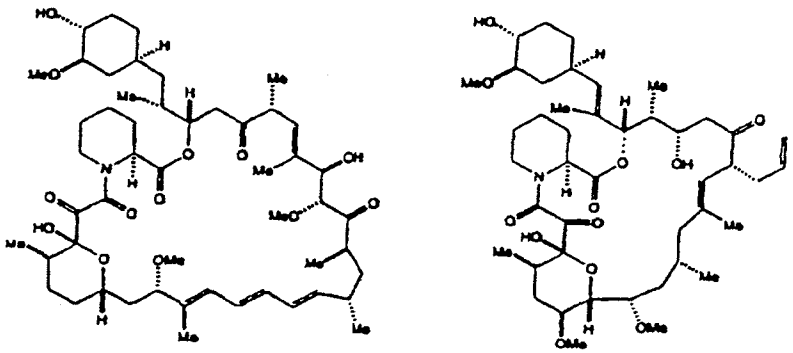

Figure 1 The chemical structures of rapamycin (*left*) and FK506 (*right*).

(11). However, the drug received more serious consideration as an immunosuppressant nearly a decade later, coincident with the discovery of FK506 at the Fujisawa Pharmaceutical Laboratories. FK506 ($C_{44}H_{69}NO_{12}$, Figure 1) was identified in a strain of *Streptomyces tsukubaensis* isolated from a soil sample taken from the Tsukuba region of Northern Japan during a screen for natural products that inhibit IL-2 production (12). FK506 was shown to be a powerful immunosuppressive agent as measured by its inhibitory effects in several immune function assays, including alloantigen-induced proliferation of lymphocytes, generation of cytotoxic T lymphocytes, expression of IL-2 receptors, and the production of T lymphocyte–derived lymphokines such as IL-2, IL-3, and IFN-γ (13). Interest in the therapeutic potential of FK506 mounted when it was discovered to be 100-fold more potent than cyclosporin A (CsA), the mainstay of transplant rejection drugs (13). The observations that the macrolactam rings of FK506 and RAP both contain the distinctive hemiketal-masked α, β-diketopipecolic acid amidic component (Figure 1) (14) and that FK506 is a more potent immunosuppressant than CsA provoked a resurgence of interest in RAP as a candidate immunosuppressive drug (15).

Although RAP and FK506 bind to the same family of intracellular receptors (see below), studies of the immunosuppressive activities of both agents in vitro (16, 17) quickly proved that the mechanism of RAP action is distinct from that of FK506. While the development of RAP as a therapeutic agent has lagged behind that of FK506, studies in both rodent and larger animal models have confirmed that RAP is a potent immunosuppressive compound. The therapeutic indices of RAP in various species, arranged in the order of greatest to least, are: mouse and rat, pig, monkey, and dog (18; reviewed in 19). In rodents, RAP is a potent inhibitor of the rejection of both xeno- (20) and allogeneic (21) skin grafts and, in highly histoincompatible heart grafts (18, 22), it significantly outperforms FK506 in terms of both potency and graft survival time (22). In histoincompatible renal transplants in pigs (18, 23, 24), rats (25), and primates (26), RAP has also demonstrated its effectiveness in preventing rejection. In mice, it is a more potent inhibitor of the graft-versus-host reaction than either FK506 or CsA. In the same species, RAP prevents host-versus-graft disease with higher potency than CsA, but not FK506 (27). Further studies have confirmed the initial finding (11) that RAP protects against autoimmune disease in rodent models. In the MLR/lpr mouse model of human systemic lupus erythematosus, RAP prolongs survival and prevents progression of the glomerulonephritis associated with the disease. In the nonobese diabetic mouse model of human autoimmune insulin-dependent diabetes mellitus, RAP prevents the onset of the disease whereas CsA is without effect (28). RAP also inhibits developing and established adjuvant-induced arthritis in the rat and has

significant inhibitory effects on the incidence and severity of collagen-induced arthritis in mice (28).

Relative to the severe renal side effects observed during therapy with CsA and FK506, the nephrotoxicity caused by RAP treatment is negligible (18, 28–30). However, it is not without serious side effects, particularly in larger animals. As observed with FK506 (31), RAP is extraordinarily toxic when administered to dogs, and even short, nonimmunosuppressive dosing regimens severely disturb gastrointestinal functions, producing diarrhea, vomiting, severe ulceration, and vasculitis from the mouth to the colon (18). Because FK506 and RAP inhibit different target proteins (see below), this strikingly similar toxicity profile in dogs is intriguing. The gastrointestinal toxicity may be due to a chemical property shared by the two compounds or to the fact that both drugs bind to the same intracellular receptors, disrupting an as-yet-undefined FKBP-dependent process in the dog gut. The gastrointestinal side effects are not species-specific, as severe vomiting and intestinal vasculitis are also observed in baboons treated with RAP (26). Another adverse side effect associated with RAP-treatment is testicular atrophy, observed in both mice and cynomolgus monkeys (32). Interestingly, relative to all other human tissues examined, the mRNA encoding the target of the FKBP · RAP complex, mTOR, is very highly expressed in testis (33), suggesting a functional role in testicular physiology.

As studies with FK506 and CsA have shown, toxicity, or the lack thereof, in animals does not necessarily extrapolate to humans (34, 35), and despite its significant adverse side effects, RAP has exhibited sufficient therapeutic potential to proceed to Phase II clinical trials (36–38). Consideration has also been given to the use of RAP in combination with CsA, which may result in additive or even synergistic immunosuppressive effects, with the attendant reduction in individual drug dosage leading to a decreased incidence of adverse side effects (39). The toxicity associated with CsA, FK506, or RAP therapy is not unexpected. The immunophilin complexes containing CsA, FK506, or RAP inhibit the functions of specific but ubiquitously expressed target proteins in mammalian tissues. The profiles of therapeutic and toxic effects observed with each drug likely reflect the abundance and functions of the cognate target proteins, as well as pharmacodynamic parameters such as each drug's ability to penetrate cellular membranes, the concentrations of different immunophilins within various cell types, and the relative affinities of the various immunophilin · drug complexes for their target proteins.

MOLECULAR MECHANISM OF RAPAMYCIN ACTION

The groundwork for understanding the molecular basis of RAP-induced immunosuppression was provided by a remarkable series of mechanistic insights

into the cellular pharmacology of CsA and FK506. CsA and FK506 both inhibit the same subset of Ca^{2+}-associated activation pathways (40), exert their inhibitory effects during the G_0-to-G_1 phase of T cell activation, and block expression of the same set of early lymphokine genes (41). CsA and FK506 bind to abundant, ubiquitous, and phylogenically well-conserved intracellular receptors (42, 43). The major cytosolic receptor for CsA is cyclophilin A (CyPA), an 18-kDa protein (44), while the major FK506 binding protein (FKBP) is a 12-kDa cytosolic protein termed FKBP12 (45, 46). Both CyPA (47, 48) and FKBP12 (45, 46) are enzymes, termed peptidyl-prolyl isomerases (PPIases), that catalyze *cis-trans* isomerization of peptidyl-prolyl bonds in peptides and proteins. FKBP12 prefers substrates with hydrophobic amino acids immediately preceding the proline, while CyPA is a more promiscuous enzyme (49). Although the PPIase activities of CyPA and FKBP12 are inhibited by CsA (47, 48) and FK506 (45, 46), respectively, inhibition of PPIase activity is unrelated to immunosuppression (50–52). These observations suggested a gain-of-function model in which FK506 and CsA are inactive alone but serve as co-drugs with their cognate immunophilins, forming active complexes that inhibit a Ca^{2+}-dependent signal transduction event (53). Subsequently, it was shown that the CyPA · CsA (54) and the FKBP12 · FK506 (55) complexes bind to and inhibit the same target protein, the Ca^{2+}-dependent serine-threonine phosphatase, calcineurin (CaN), a critical component of the TcR-linked signal transduction pathway leading to cytokine gene transcription (56, 57). CaN is activated by the increase in cytoplasmic free Ca^{2+} that results from TcR engagement. A target, either direct or indirect, for the activated phosphatase is the phosphorylated cytoplasmic subunit of the T cell–specific transcription factor, nuclear factor of activated T cells (NFAT). The dephosphorylated cytoplasmic NFAT subunit is free to translocate to the nucleus and associate with a nuclear subunit to form the fully active NFAT complex, an essential component of the transcriptional apparatus required for expression of the IL-2 and other cytokine genes. Thus, by interfering with the TcR-mediated activation of CaN, both CsA and FK506 block transcription of the IL-2 gene in T lymphocytes (for reviews, see 58–60).

The Intracellular Receptors for RAP

RAP and FK506 bind to the same family of intracellular receptors, termed FK506 binding proteins (FKBPs). Structural studies have shown that FK506 has two domains—a domain bound by FKBP and an effector domain that, together with FKBP, forms a composite surface that interacts with CaN (reviewed in 61; 62). Like FK506, RAP also has two domains—an effector domain forming a composite surface with FKBP that interacts with the mammalian target of RAP, mTOR (see below), as well as a binding domain that mediates the

interaction with FKBP. The FKBP-binding domain is conserved in FK506 and RAP, providing a chemical basis for the mutual antagonism exerted by the two molecules in intact cells (16, 63).

The crystal structure of the human FKBP12 · RAP complex has been solved and shows the pipecolinyl ring of RAP buried deeply in the hydrophobic cavity located between the α-helix and β-sheet of FKBP12 (64). The common chemical elements of RAP and FK506 (Figure 1), which include the pipecolinyl ring, C1 ester, pyranose ring, and the C8 and C9 carbonyls, adopt superimposable conformations in the FKBP12 · RAP and FKBP12 · FK506 complexes (64). In contrast to FK506, which undergoes a dramatic conformational change upon binding FKBP12 (65), the three-dimensional structure of RAP complexed to FKBP12 is almost identical to its conformation in the free crystalline state (64). Thus, relative to FK506, RAP is in an energetically favorable conformation for binding to FKBP12, providing an explanation for its two-fold greater affinity.

The human FKBP family is currently comprised of seven members, whose characteristics are summarized in Table 1. Exhaustive reviews on FKBPs can be found elsewhere (66, 67). All FKBPs bind RAP with greater affinity than FK506, with the exception of FKBP38 (FKBP-related 38-kDa protein) which does not bind the immunosuppressants but is included in the family because of the similarity of its sequence (68). The PPIase activity of all FKBPs is inhibited by RAP and FK506. Because the drug-binding domain overlaps with the PPIase active site, inhibition of PPIase activity by RAP or FK506 can be taken as a measure of the affinity of a particular FKBP for the drug. Among the FKBPs, FKBP25 is most selective for RAP relative to FK506 (69). Despite this selectivity, FKBP25 does not mediate the RAP-sensitivity of mast cells (70), suggesting that the FKBP25 · RAP complex does not interact with mTOR (see below) in intact cells. To date, two FKBP isoforms, FKBP12 and FKBP12.6, have been shown to bind mTOR in the presence of RAP, although the FKBP12 · RAP complex appears to be superior in this regard (71).

There is no evidence linking the physiological function of the FKBPs to their inhibitory functions in the presence of RAP. FKBP12 and FKBP12.6, which associate with mTOR in the presence of RAP, normally interact with the calcium release channel (CRC)/ryanodine receptors (RyR) of the terminal cisternae of skeletal muscle and heart muscle sarcoplasmic reticulum, respectively, and they are required for proper channel function (72–76). The ryanodine receptors in skeletal muscle (isoform 1, RyR-1) and heart muscle (isoform 2, RyR-2) are the largest ion channel complexes known (MR, 2.3×10^6 daltons), and they play an important role in release of Ca^{2+} during excitation-contraction coupling. The skeletal muscle CRC can be represented as (RyR-1 protomer)$_4$(FKBP12)$_4$, while the cardiac muscle CRC can be represented

Table 1 The family of human FKBPs

FKBP	MW (kDa)	% Identity to FKBP12	Affinity (nM) RAP	Affinity (nM) FK-506	PPIase activity[g]	Binds mTOR[h]	Inhibition of CaN[i](IC50)	Physiological association
FKBP12	11.8	100	0.2 (K_d)	0.6 (K_d)	yes	yes	8 nM	RyR-1[k]; IP_3R^l
FKBP12.6[a]	11.6	83	0.2 (K_d)[d]	0.5 (K_d)	yes	yes	8 nM	RyR-2[m]
FKBP13	13.3	50	ND[e]	55.0 (K_i)	yes	ND	30 μM	Lumen of ER
FKBP25	25.0	40	0.9 (K_i)	160 (K_i)	yes	ND	> 50 μM	Casein kinase II; nucleolin
FKBP38[n]	38.3	33	no binding	no binding	no	NA[j]	NA[j]	
FKBP51[b]	51.2	50	29 (IC_{50})[f]	166 (IC_{50})[f]	yes	ND	3 μM	
FKBP52[c]	51.8	53	8 (K_i)	10 (K_i)	yes	ND	30 μM	Glucocorticoid receptor; hsp9C

[a]An alternative splice product of the FKBP12.6 mRNA encodes an 8.8 kDa FKBP-related protein (133). [b]This human cDNA has recently been cloned (Baughman G et al, unpublished results). [c]Also known as hsp56, FKBP59, p59, hsp59. [d]Based upon the K_d for FK-506 and adjusted for RAP's greater ability to inhibit PPIase activity (71). [e]These values are for murine FKBP51 (134). [f]RAP and FK-506 inhibit PPIase activity in all FKBPs. [g]When the indicated FKBP is complexed with RAP. [h]Not applicable because FKBP38 (FKBP-related 38 kDa protein) does not bind RAP or FK-506. [i]When the indicated FKBP is complexed with FK-506 (71). [j]Not determined. [k]Ryanodine receptor calcium release channel in skeletal muscle sarcoplasmic reticulum. [l]The inositol triphosphate receptor on the endoplasmic reticulum. [m]Isoform 1 of the ryanodine receptor calcium release channel in cardiac muscle sarcoplasmic reticulum. [n]The 38 kDa mass is based upon translation of the ORF in an isolated cDNA.

as (RyR-2 protomer)$_4$(FKBP12.6)$_4$ (73, 74). Both RAP and FK506 displace FKBP12 from RyR-1 (73), but it is unlikely that at immunosuppressive doses any displacement actually occurs in vivo, since the concentration of FKBP12 in skeletal muscle myoplasm is about 3 μM (74). This would effectively buffer any disruption of the channel complex by either drug. Recently, it has been shown that the IP_3R CRC, a relative of the RyR CRC, is also associated with and modulated by FKBP12 (77). RAP and FK506, but not CsA, can dissociate FKBP12 from the channel, rendering it leaky to Ca^{2+} (77). Again, it is unlikely that immunosuppressive doses of RAP would have an effect on the IP3R CRC in T cells. Only 3–5% FKBP occupancy is required to prevent activation of T cells, and, as in muscle cells, the high intracellular concentration of FKBP (6 to 7 μM) (52) would buffer any effect that RAP might have on the IP_3R-associated FKBP12.

The Targets of the FKBP · RAP Complex in Yeast

Studies in yeast, in which two independent groups first identified the target of the FKBP · RAP complex, have made enormous contributions to our understanding of the mechanism of RAP action in mammalian cells. The extreme growth-sensitivity of *S. cerevisiae* to RAP (IC_{50} = 0.1 μg/ml) allowed for selection of RAP-resistant mutants that identified genes mediating RAP sensitivity. Mutant alleles of three genes *FKB1*, *TOR1 (DRR1)*, and *TOR2 (DRR2)* (TOR: target of RAP; DRR: dominant RAP resistant), were found to confer resistance to RAP (78–82). Mutations in *FKB1* (encoding FKBP12) were recessive while the *TOR1* and *TOR2* mutations isolated in these studies were dominant or semi-dominant (80–82). *TOR1*-disruptants exhibit a mild phenotype, growing 10–15% more slowly than wild-type strains (82). When grown in the presence of RAP, the *TOR1*-disruptants arrest growth in early G_1 within one generation. The TOR2 disruption confers a lethal growth defect with cells arresting not only in G_1 but randomly throughout the cell cycle. Strains disrupted for both *TOR1* and *TOR2* arrest growth in G_1 within one generation, mimicking the phenotype of wild-type yeast grown in the presence of RAP. The G_1 arrest phenotype of the *TOR1/TOR2* double-disruptant indicates that RAP inhibits both the TOR1 and TOR2 proteins (82). The presence of either TOR1 or TOR2 is sufficient to allow yeast cells to progress through G_1, indicating that either protein can execute the essential G_1 function. TOR2 has two functions, a RAP-sensitive G_1 function complemented by TOR1 and a RAP-insensitive essential function not complemented by TOR1 (80). The observation that placement of a *TOR2*-disruption in a *TOR1*-mutant, RAP-resistant background confers a lethal phenotype (83) does support the initial suggestion (80) that TOR2 has two functions.

TOR1 and TOR2 are proteins with 2470 (281.2 kDa) and 2474 (282 kDa) amino acid residues, respectively, which lack obvious signal sequences or

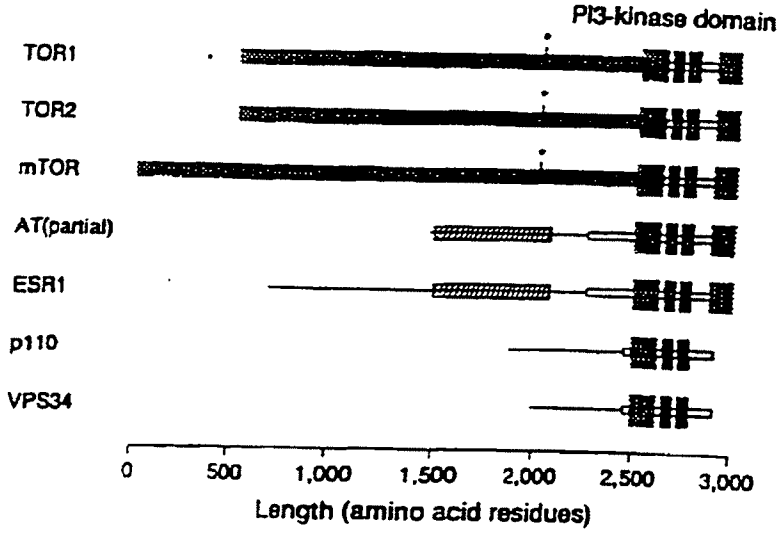

Figure 2 A schematic representation of TOR family members and related proteins. The *asterisk* marks the location of the critical serine residues, Ser^{2035}, Ser^{1972}, and Ser^{1975}, in mTOR, TOR1, and TOR2, respectively. Similarly shaded boxes indicate regions of high homology among PI 3-kinase family members.

transmembrane domains (80, 81). The two proteins have 67% amino acid identity and 80% similarity overall (84). The N-terminal segments of both proteins are unrelated to any protein currently in the databases. The C-terminal regions of TOR1 and TOR2 are homologous to a growing family of signaling proteins. These include (i) the p110 catalytic subunit of bovine phosphatidylinositol 3-kinase (PI 3-kinase), (ii) a PI 3-kinase (VPS34) involved in targeting soluble hydrolases to vacuoles in *S. cerevisiae* (85), (iii) a protein (ESR1, MEC1) required for the repair of damaged DNA and for meiotic recombination in *S. cerevisiae* (86), and (iv) the protein encoded by the human *AT* gene, which is mutated in the autosomal recessive disorder ataxia telangiectasia (87). The regions of greatest homology among the six proteins (amino acids 2123–2296 of TOR1, 2127–2300 of TOR2, 801–935 of p110, 623–751 of VPS34, 2078–2245 of ESR1, and 1368–1543 of the protein encoded by the partial AT cDNA clone) span a putative "lipid kinase motif" containing residues conserved in the ATP-binding domains of PI 3- and PI 4-kinases as well as certain protein kinases (Figure 2).

The lipid kinase domains in TOR1 and TOR2 are functionally interchangeable (82). Thus, the different phenotypes of the TOR1- and TOR2-disrupted yeast reflect functional differences in the amino-terminal regions of each protein.

All TOR1 and TOR2 alleles that have been cloned from RAP-resistant yeast strains are missense mutants that alter the same serine residue just upstream of the lipid kinase domain (80–82, 84). The FKBP · RAP complex binds a 196 amino acid fragment of TOR2 (amino acids 1886–2081), an interaction abolished by mutations of Ser^{1975} (88). Likewise, the binding domain of TOR1 has been mapped to a small region surrounding Ser^{1972}, and mutations to any residue other than alanine prevent binding of FKBP· RAP and confer resistance to RAP (83). Because alanine is a potential mimic of a nonphosphorylated serine residue, these results indicate that phosphorylation of Ser^{1972} is not required for FKBP · RAP binding, and they also argue against an earlier proposal that *phosphorylation of* $Ser^{1972/1975}$, *present in a consensus protein kinase C site*, may be required for binding FKBP12 · RAP (82).

Although it has not yet been demonstrated that TOR1 and TOR2 actually possess kinase activities, genetic evidence strongly suggests that intact lipid kinase domains are required for their G_1 function. The introduction of mutations analogous to those known to abolish the lipid kinase activities of VPS34 and mammalian p110 into the lipid kinase domains of the dominant RAP-resistant *TOR1* or *TOR2* alleles abrogates their ability to confer resistance to RAP (83). The reversion to RAP-sensitivity of these strains demonstrates that functional kinase domains in TOR1 and TOR2 are necessary for their G_1 function. When overexpressed in a wild-type background, "kinase dead" *TOR1* mutants confer a dominant negative phenotype, resulting in G_1 arrest (83). This finding suggests that the overproduced, mutant TOR1 protein is nonproductively interacting with normal G_1 targets of the wild-type TOR1 kinase domain. The essential non-G_1 function of TOR2 also requires an intact kinase domain. These genetic data support a model (83) which proposes that the G_1 target of the TOR kinase domains binds to the C-terminus of the TOR proteins, an interaction blocked by FKBP12 · RAP. The model further suggests that the target substrate for TOR2's essential viability function binds to a different region, presumably located in the polymorphic N-terminus of TOR2, and its access to TOR2 is not blocked by FKBP12 · RAP.

Recent studies suggest that the TOR proteins function in a signal transduction pathway that coordinates the availability of essential nutrients with progression through the cell cycle. Even when grown on rich medium, yeast cells depleted of TOR function, either by RAP-treatment or by disruption of both *TOR* genes, display a phenotype similar to that of cells entering G_0 during the starvation response. For example, both nutrient deprivation and treatment with RAP cause a rapid and acute reduction in initiation of translation, an accumulation of glycogen, an increase in vacuole size, a greatly increased transcription of known marker genes for nutrient starvation, and an arrest of yeast cells with 1N

DNA (89). The putative TOR-restriction point has been mapped to a point prior to the α-mating factor arrest point, START (89). TOR-depleted cells arrest in early G_1 because they are unable to synthesize CLN3, a cyclin required for G_1 progression. CLN3 functions, in part, as a transcriptional activator of genes expressed later in G_1, including CLN1, CLN2, ORFD, HCS26, and CLB5, all of which encode known or candidate cyclins. CLN3 expression is regulated by the translational initiation factor eIF-4E. When the CLN3 ORF is fused to eIF-4E-independent 5′ translational regulatory sequences, RAP-induced G_1 arrest is repressed (89). These results indicate that TOR is a signaling molecule required for eIF-4E-dependent translation when nutrient conditions are favorable for G_1-phase progression.

The Mammalian Target of Rapamycin (mTOR)

Five groups, working independently, identified the mammalian protein target of the FKBP12 · RAP complex. Homologous high molecular weight proteins were isolated from various mammalian sources including bovine brain (FKBP-RAP-associated protein, FRAP) (33), rat brain (RAP and FKBP12 target, RAFT; mammalian target of RAP, mTOR) (90, 91), and human lymphocyte (Sirolimus effector protein, SEP; RAP target, RAPT) (92, 93). In keeping with the precedent yeast nomenclature, we refer to the protein as mTOR. Based upon its migration in denaturing gels, the purified mTOR protein has a molecular weight greater than 200 kDa (33, 90–92). Two observations helped to confirm that mTOR mediates the inhibitory effects of RAP in mammalian cells. First, two structural analogs of RAP (16-keto-RAP and 25, 26 iso-RAP) were identified that bind with high affinity to FKBP12 but which are 100-fold less potent inhibitors of G_1 progression in MG-63 osteosarcoma cells. The FKBP12 complexes with 16-keto-RAP and 25, 26 iso-RAP complexes bind mTOR poorly, if at all (33). Second, little or no mTOR is bound by the FKBP12 · RAP complex in extracts prepared from mutant murine T cell (YAC) lines selected for RAP-resistance (91, 94). Extracts prepared from a RAP-sensitive revertant derived from one of the RAP-resistant T cell lines show wild-type levels of mTOR bound to the FKBP12 · RAP complex (91). Thus, sensitivity of T cells to RAP correlates with binding of mTOR to the FKBP12 · RAP complex.

Microsequencing of the purified mTOR protein enabled the cloning of the complete cDNAs encoding both the human (33) and rat homologs (90, 91). The open reading frames encode 2549 amino acid proteins that have calculated molecular weights of 289 kDa. The protein product of the in vitro–translated rat mTOR cDNA binds to the FKBP12 · RAP complex—formal validation that the cloned cDNA encodes a direct ligand for this complex (91). The mRNA of mTOR is ubiquitously expressed in human tissues with the highest levels found in testis and significant expression found in skeletal muscle (33, 93). Both

human and rat mTOR are slightly more similar to yeast TOR2 (46% identity) than to TOR1 (44% identity), with the greatest similarity (65% identity) found in the C-terminal 600 amino acids of the three proteins. This region of mTOR contains the lipid kinase motif (amino acids 2186–2359) found in the yeast TORs. There are other regions, particularly in the amino terminal portions of the proteins, where there is little or no homology between the yeast TORs and mTOR. Thus, although it is unclear whether mTOR is the functional equivalent of yeast TOR1 or TOR2, the high degree of amino acid sequence identity in the lipid kinase domain suggests that these proteins have similar enzymatic activities. To date, in vitro kinase assays have failed to detect lipid kinase activity in mTOR although a serine autophosphorylation activity has been reported (83).

As shown with the yeast TOR proteins (88), only a small portion of mTOR mediates binding to the FKBP12 · RAP complex. In mTOR, the binding domain has been mapped to amino acids 2025–2114 (95), a 90 amino acid region just upstream of the lipid kinase domain containing a critical serine residue (Ser^{2035}) homologous to the $Ser^{1972/1975}$ in the yeast TOR proteins. As in TOR1, mutation of Ser^{2035} to residues other than alanine abrogates binding of mTOR to FKBP12 · RAP (93, 95), indicating that phosphorylation of Ser^{2035} is not required for binding by FKBP12 · RAP and that Ser^{2035} lies in a structurally critical region that is disturbed when replaced by any residue larger than alanine.

BIOCHEMICAL ACTIONS OF RAPAMYCIN IN MAMMALIAN CELLS

Anti-Proliferative Effects of RAP in Lymphoid and Nonlymphoid Cells

RAP is a potent inhibitor of the growth of most hemopoietic and lymphoid cell lines in vitro (17). Maximal growth-inhibitory effects are usually observed at concentrations of the drug equal to or less than 10 nM. It is particularly striking that RAP suppresses the cytokine-driven proliferation of both nontransformed lymphoid cell lines and the continuous growth of several leukemic cell lines, with virtually equal potency. The increase in doubling time of the cells induced by the drug is accompanied by a dramatic increase in the proportion of G_1-phase cells, which is consistent with the model that the FKBP12 · RAP complex interferes specifically with the progression of G_1-phase cells into S phase (96, 97). The growth-inhibitory effect of RAP on lymphoid and other cell types is effectively antagonized by FK506 (16). Conversely, the ability of FK506 to inhibit antigen-dependent IL-2 production is reversed by RAP. The reciprocal antagonism between FK506 and RAP is explained by the competitive interactions of these drugs with a common intracellular receptor FKBP12.

In contrast to the broadly anti-proliferative effects of RAP on bone marrow–derived cells, the efficacy of RAP as an inhibitor of mesenchymal or epithelial cell growth is variable. For example, the serum-dependent growth of Swiss 3T3 mouse fibroblasts is only marginally affected by nanomolar concentrations of RAP (98), whereas RAP profoundly inhibits the proliferation of MG-63 osteosarcoma cells under the same culture conditions (97). The variable response to RAP may reflect physiologic differences in the extent to which redundant signaling pathways compensate for the loss of mTOR function in different cell lineages. Alternatively, certain established cell lines may have acquired abnormalities in cell-cycle regulation that effectively override the putative G_1-phase checkpoint governed by mTOR.

Other Cellular Actions of RAP

Although the immunosuppressive activity of RAP is commonly attributed to its antiproliferative effect on lymphoid cells, this drug affects a number of other cellular functions that may contribute to its ability to suppress immune responses in vivo. Studies performed with the murine T cell lymphoma, YAC-1, have shown that RAP inhibits IL-1-stimulated production of interferon (IFN)-γ as well as IFN-γ-induced expression of Ly6E antigen on the cell surface (94, 99, 100). Both of these actions are antagonized by FK506, suggesting that they are dependent upon the formation of FKBP12 · RAP complexes in YAC-1 cells. Furthermore, YAC-1 somatic mutants selected for resistance to the growth-inhibitory effect of RAP were correspondingly resistant to the suppressive actions of RAP on cytokine-driven production of IFN-γ and expression of Ly6E (94). As mentioned previously, the resistant phenotype of these YAC-1 clones is apparently explained by a mutational event(s) that leads to a decreased affinity of mTOR for the FKBP12 · RAP complex. These results suggest that, in addition to its cell-cycle regulatory function, mTOR participates in the signal transduction pathways that mediate IL-1- and IFNγ-stimulated responses in YAC-1 cells.

Ligation of the CD28 receptor on T cells provides an important costimulatory signal for antigen-dependent cytokine production by helper T-lymphocytes. The signal transduction pathway initiated by the interaction of CD28 with its ligands B7-1 and B7-2 remains obscure. In contrast to signaling from the TCR, signal propagation from the CD28 receptor is not inhibited by CsA or FK506. Conversely, signaling through CD28, but not the TCR, is sensitive to RAP (101). The CD28 response element in the IL-2 promoter region contains a nucleotide sequence that resembles a NFκB-binding site, suggesting that CD28 promotes transcription of the IL-2 gene through the activation of members of the Rel family of transcription factors (102). Stimulation of Jurkat or human peripheral blood T cells with phorbol ester induces the nuclear translocation of the NFκB

family member c-Rel, and both the rate and magnitude of this response are enhanced by costimulation through CD28 (103). The increase in intranuclear c-Rel is accompanied by a sustained downregulation of the inhibitory IκB-α subunit in the cytoplasm. Both responses are inhibited by RAP, but not by CsA, suggesting that the RAP-sensitive target protein, mTOR, is involved in the coupling mechanism between CD28 receptor stimulation and c-Rel-dependent transcription. The impact of RAP on CD28 signaling clearly warrants further investigation, as this drug effect could represent a crucial component of the overall immunosuppressive action of RAP in vivo.

Activation of p70S6 Kinase

The potent antiproliferative effects of RAP prompted speculation that the pharmacologically active immunophilin · drug complex targets a protein kinase involved in the relay of mitogenic signals from the cytoplasm to the nucleus. The most obvious candidate was a component of the Ras-to-MAP-kinase (MAPK) signaling cascade (i.e. Raf, MAPK kinase, or MAPK itself). However, subsequent studies failed to uncover any detectable effect of RAP on Ras signaling in mammalian cells (98; RT Abraham, unpublished data). Instead, the drug was shown to disrupt a Ras-independent signal transduction pathway required for activation of the 70-kDa S6 protein kinase ($p70^{S6K}$). Activation of $p70^{S6K}$ is apparently a universal response of mammalian cells to mitogenic stimuli. In activated T cells, the activity of $p70^{S6K}$ increases within 10 min of addition of IL-2, and activation is maximal after 40–60 min of stimulation (104). This response is accompanied by a decrease in the electrophoretic mobility of $p70^{S6K}$, which likely reflects an increase in the phosphorylation of the enzyme. Pretreatment with RAP abolishes both the shift in electrophoretic mobility and the increase in catalytic activity of $p70^{S6K}$ induced by growth factors in T cells as well as in all other mammalian cell types (98, 104–106). The drug concentrations needed to abolish activation of $p70^{S6K}$ are identical to those required to block the progression of mitogen-stimulated cells through G_1-phase. The suppressive effect of RAP on activity of $p70^{S6K}$ is reversed in the presence of excess FK506, indicating that this action is dependent on the binding of RAP to an FKBP, probably FKBP12. A striking observation is that the addition of RAP to cells at any time after exposure to growth factor leads to a rapid decline in activity of $p70^{S6K}$ to (or even below) the basal level observed in quiescent cells. The ability of RAP to inhibit preactivated $p70^{S6K}$ suggests that the FKBP12 · RAP complex disrupts a constitutive signaling event required for both the generation and maintenance of the activated form of $p70^{S6K}$.

The biochemical results described above are consistent with the idea that the FKBP12 · RAP complex interferes directly or indirectly with the function of a

$p70^{S6K}$-activating protein kinase in mitogen-stimulated cells. As $p70^{S6K}$ itself is not sensitive to RAP (98), the drug's site of action is thought to lie at an upstream point in the signaling pathway that links stimulation of growth factor receptors to activation of $p70^{S6K}$. It is becoming increasingly evident that $p70^{S6K}$ does not lie in a simple, linear signaling cascade, and recent studies suggest that at least two $p70^{S6K}$-activating protein kinases regulate the state of phosphorylation of the enzyme in growth factor-stimulated cells (107, 108). The carboxy terminus of $p70^{S6K}$ contains a 25 amino acid segment whose sequence suggests that it functions as an autoinhibitory pseudosubstrate domain. According to the current model, phosphorylation of the carboxy-terminal regulatory domain at multiple serine and threonine residues relieves an inhibitory constraint on the catalytic domain and allows activation of the enzyme. However, activation of $p70^{S6K}$ also requires an additional stimulatory input mediated through a highly acidic stretch of amino acids in the amino-terminal region (residues 29–46 in the rat protein) of $p70^{S6K}$. Mutational analyses of $p70^{S6K}$ indicate that it is the essential activating signal delivered through the amino-terminal segment that is blocked by RAP (107, 108). Although treatment with RAP has no effect on the mitogen-induced phosphorylation of the carboxy-terminal pseudosubstrate domain, it inhibits phosphorylation elsewhere in $p70^{S6K}$. The simplest interpretation of these results is that the amino-terminal acidic region mediates the interaction of $p70^{S6K}$ kinase with a RAP-sensitive, $p70^{S6K}$-activating kinase. If correct, this model places the RAP target protein, mTOR, in a protein-serine-threonine kinase cascade leading to activation of $p70^{S6K}$.

Does inhibition of $p70^{S6K}$ explain the growth-suppressive action of RAP in lymphoid cells? Unfortunately, a straightforward answer to this question is not yet available. Microinjection of neutralizing anti-$p70^{S6K}$ antibodies into rat embryo fibroblasts blocks serum-induced G_1- to S-phase progression, suggesting that this enzyme plays a key role in transduction of mitogenic signals (109). However, the universality of this conclusion is challenged by experiments with RAP itself. Whereas RAP blocks growth factor-dependent activation of $p70^{S6K}$ with virtually equal potency in both lymphoid and nonlymphoid cells, the efficacy of RAP as a growth inhibitor is relatively unpredictable, particularly in nonhemopoietic cell lines. Thus, if $p70^{S6K}$ executes a critical function required for S-phase entry, redundant signaling pathways must compensate for the loss of $p70^{S6K}$ activity in cells that are relatively insensitive to the growth-inhibitory action of RAP.

Effects of RAP on Protein Synthesis

The progression of mitogen-stimulated cells through G_1 phase is contingent upon regulated alterations in both the transcription of specific genes and the translation of certain mRNA transcripts. Initiation of translation is generally

the rate-limiting step in protein synthesis and therefore represents a logical point of regulation (110, 111). Eukaryotic mRNAs bear a 7-methylguanylate (m^7GTP) cap at the 5′-terminus, and this cap region serves as the recognition site for the eukaryotic initiation factor (eIF)-4F complex. Two components of the multisubunit eIF-4F complex are the ATP-dependent helicase eIF-4A and the mRNA cap-binding protein eIF-4E. Initiation involves the recognition of the m^7GTP cap by eIF-4E, followed by the eIF-4A-dependent unwinding of 5′ secondary structure in the target mRNA. These events are thought to facilitate binding of the methionyl tRNA-charged 40S ribosomal subunit to the mRNA. This model predicts that the degree to which individual mRNA transcripts depend on the eIF-4F complex for initiation of translation varies directly with the complexity of the secondary structure at the 5′-terminus of the mRNA. Thus, the translation of particular mRNAs will be variably sensitive to regulatory alterations in the function of eIF-4F. As might be expected, a major mechanism by which eIF-4F is regulated involves the phosphorylation of specific components of the eIF-4F complex.

An important target of protein kinases involved in controlling initiation of translation is the cap-binding protein eIF-4E. Recent studies have shown that the binding activity of eIF-4E is regulated by its interaction with PHAS-I, a heat-stable inhibitor of eIF-4E function (112, 113). In resting cells, PHAS-I is tightly bound to eIF-4E and thereby inhibits the ability of eIF-4E to trigger eIF-4F-dependent initiation. Stimulation of cells with growth factors, including serum, insulin, or IL-2, leads to the hyperphosphorylation of PHAS-I, and release of its associated eIF-4E. Hence, mitogenic stimuli are capable of stimulating rapid increases in initiation of translation by activating protein kinases that phosphorylate PHAS-I. Although PHAS-I is an excellent in vitro substrate for MAP kinase, the crucial phosphorylation events leading to the release of eIF-4E are apparently not performed by this enzyme in intact cells (114). Rather, the PHAS-I kinase responsible for disinhibition of eIF-4E in vivo lies within a RAP-sensitive signaling pathway. Pretreatment of activated T cells or 3T3-L1 fibroblasts with RAP abolishes both the increase in phosphorylation of PHAS-I and the release of active eIF-4E induced by IL-2 or insulin (114; GJ Brunn, RT Abraham, J Lawrence, unpublished observations). These results strongly suggest that an mTOR-regulated protein kinase controls eIF-4E-dependent initiation of translation in mammalian cells. These findings are similar to those found in yeast. Thus, studies with RAP as a probe have uncovered a general mechanism for the control of protein synthesis in eukaryotic cells.

The inhibitory effects of RAP on phosphorylation of PHAS-I and the function of eIF-4E provide a rational explanation for the selective, rather than global, suppressive actions of RAP on mitogen-induced protein synthesis. For example,

RAP specifically interferes with the synthesis of proteins from a class of mRNAs bearing polypyrimidine tracts at their 5′-termini (115, 116). Included among these polypyrimidine-containing mRNAs are transcripts that encode ribosomal proteins and elongation factors, i.e. components of the protein synthetic machinery itself. In many cases, the 5′-untranslated regions of these mRNAs also contain nucleotide sequences predicted to have considerable secondary structure, thereby making initiation of translation strongly dependent on eIF-4E. Therefore, the translation of this class of mRNAs should be particularly sensitive to RAP.

In summary, accumulating evidence suggests that the growth-inhibitory mechanism of RAP is inextricably linked to the machinery that controls the inducible synthesis of specific proteins in growth factor–stimulated cells. If this model is correct, the next task will be to identify the mRNA transcript(s) whose translation limits the rate of passage of growth factor-stimulated lymphoid cells through the mid/late G_1-phase checkpoint defined by RAP. This effort will be greatly facilitated by an understanding of the effect of RAP on the biochemical machinery that controls the passage of cycling cells through G_1 and into S phase: the G_1 cyclins and their associated cyclin-dependent kinases (cdks).

Effect of RAP on G_1 Cyclin-cdk Activities

The progression of growth factor-stimulated mammalian cells from G_1 to M phase is orchestrated by the precisely timed activation and inactivation of a series of cyclin-associated protein kinase activities (see 117–121 for reviews). The sequence and timing of activation of each cyclin-cdk complex is controlled in part by cell-cycle checkpoints, which ensure that the appropriate cyclin-cdk complex becomes active only after contingent earlier events are accurately executed (119). The G_1-specific growth-arrest state induced by RAP suggested that this drug might interfere, directly or indirectly, with the timely activation of a G_1 cyclin-cdk complex required for the entry of IL-2-stimulated, G_1-phase T cells into S phase. Progression through G_1 phase is marked by the assembly and catalytic activation of at least three sets of cyclin-cdk complexes. In IL-2-stimulated T cells, the first set of complexes to become active reflects the association of cyclin D2 with cdk4 or cdk6. This event is followed by the sequential activation of cyclin E-cdk2 and cyclin A-cdk2 complexes (116, 117). It is believed that the first two sets of cdk complexes containing cyclins D2 and E set up the conditions for passage of the cell through a restriction point in late G_1-phase (122). Thereafter, the cell is fully committed to complete one division cycle. In contrast, cyclin A-cdk2 activity, which begins to rise in late G_1 and remains elevated throughout S-phase, performs functions needed for the onset and completion of replication of DNA.

Exposure of activated T cells to RAP profoundly affects the appearance and/or functions of the G_1 cyclin-cdk activities induced by IL-2. Surprisingly little has been reported concerning the effect of RAP on the cyclin D–associated cdk complexes in this cell type. Preliminary studies suggest that RAP strongly interferes with the activation of cdks complexed with either cyclin D2 or cyclin D1 in YAC-1 T lymphoma cells or MG-63 osteosarcoma cells (97; GJ Brunn, RT Abraham, unpublished data). Conceptually more interesting results were obtained from biochemical analyses of the cyclin E-cdk2 complexes in IL-2-responsive T cells treated with RAP (123). The drug-treated cells express near-normal levels of cyclin E, and this cyclin is stoichiometrically associated with cdk2. However, the cyclin-E-cdk2 complexes assembled in the presence of RAP display essentially no detectable protein kinase activity. Although additional phosphorylation and dephosphorylation events are required to activate cyclin E-bound cdk2, the phosphorylation state of cdk2 indicated this protein kinase should be fully active in cells growth-arrested by RAP. The mechanism underlying the inhibitory effect of RAP on cyclin E-cdk2 activity remained elusive until the discovery of a novel family of negative regulators of the cell cycle termed cdk inhibitors (124).

One member of this family is Kip1, a heat-stable, titratable inhibitor of G_1 cyclin-cdk activities (125–128). Because Kip1 inhibits these activities in a stoichiometric rather than a catalytic fashion, the level of expression of Kip1 sets a threshold on the numbers of G_1 cyclin-cdk2 complexes that must be assembled before the formation of catalytically active complexes can occur (Figure 3). Recent studies using human peripheral blood T cells have shown that the initial activation step results in the expression of all three G_1 cyclins (D2, E, and, to a lesser extent, A) together with their cdk partners (cdk4, cdk6, and cdk2) (129). However, these complexes fail to become active until the cell receives a progression signal provided by IL-2 or other growth-promoting cytokines. The block to G_1 progression is explained, at least in part, by the presence of very high levels of Kip1 in activated T cells. Stimulation with IL-2 overcomes this Kip1-imposed block by inducing both a progressive decrease in the level of Kip1 protein and the assembly of additional G_1 cyclin-cdk complexes (129, 130). A provocative addendum to this regulatory scheme was provided by the recent finding that the degradation of Kip1 observed in mitogen-stimulated mammalian cells is carried out by the ubiquitin-proteasome pathway (131). This result adds Kip1 to the growing list of cell cycle–related proteins whose functions during each cycle are terminated by their timed destruction via ubiquitin-triggered proteolysis.

RAP tips the balance between activation and inhibition of cdks toward the inhibitory side by blocking the downregulation of Kip1 protein normally

provoked by IL-2 (150). Under these circumstances, the available G_1 cyclin-cdk complexes remain saturated with Kip1 and are unable to execute critical regulating functions, including the phosphorylation of the retinoblastoma protein (132), that are essential for progression through G_1. The persistent expression of Kip1 in RAP-treated T cells suggests that mTOR, the target for the FKBP12 · RAP complex, is a critical component of a signaling pathway that marks the Kip1 protein for ubiquitin-dependent proteolysis. In the light of the earlier discussion, the irony in this mechanism is obvious: mTOR apparently functions as a positive regulator during mitogen-induced protein synthesis, yet the most proximal connection to the cell cycle occurs at the level of the proteolytic destruction of Kip1. Although any model must be considered purely speculative at present, the mTOR-dependent signaling pathway may control the translation of a mRNA species whose protein product allows the ubiquitin-proteosome system to recognize and degrade Kip1. Whatever the actual mechanism, further studies with RAP as a pharmacologic probe are likely to provide some fascinating insights into the mechanism whereby occupancy of growth factor receptors at the cell surface triggers the activation of G_1 cyclin-cdk complexes in the T cell nucleus.

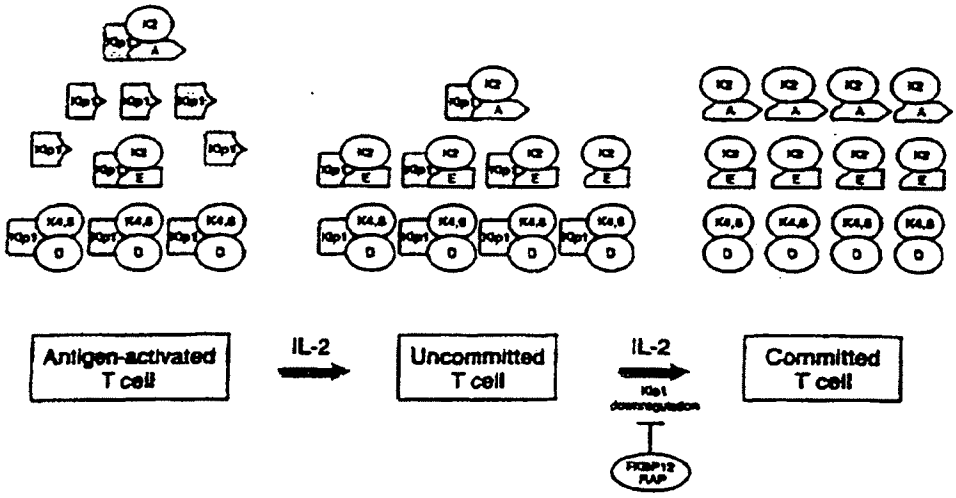

Figure 3 Role of the cdk inhibitor Kip1 in the antiproliferative mechanism of action of RAP. Kip1 functions as a stoichiometric inhibitor of G_1-cyclin-cdk complexes in activated T cells. Cyclin subunits are designated as D, E, and A, and the catalytic cdk subunits are abbreviated as K followed by the number of the appropriate cdk isoform. According to this model, RAP inhibits G_1-phase progression by blocking IL-2-dependent Kip1 downregulation.

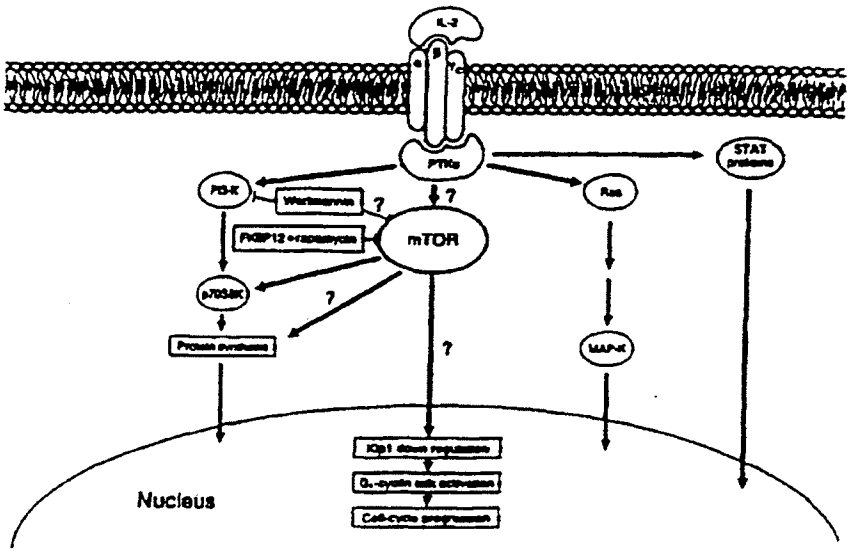

Figure 4 Schematic representation of IL-2 receptor-coupled signaling pathways. The model proposes that mTOR functions as an inducible transducer of regulatory signals for $p70^{S6K}$ activation, eukaryotic initiation factor 4E-dependent protein synthesis, and Kip1 downregulation. Interaction with FKBP12 · RAP disrupts mTOR-dependent functions. Wortmannin, an irreversible inhibitor of certain PI 3-kinase family members, also interferes with a subset of mTOR-dependent responses, including $p70^{S6K}$ activation.

CONCLUDING REMARKS

Studies of the mechanism of action of RAP are beginning to uncover a previously unrecognized signal transduction pathway that may play a general role in the control of growth of hematopoietic cells (see Figure 4 for summary). The FKBP12 · RAP complex binds to and inhibits the function of a newly defined target protein termed mTOR. The remarkable degree of sequence identity between mTOR and its yeast homologs TOR1 and TOR2 suggests that the cell-cycle regulatory function of mTOR has been highly conserved in eukaryotes. Sequence similarities in the putative catalytic domain of mTOR indicate that the RAP target protein is evolutionarily related to the phosphoinositide kinases PI 3-kinase and PI 4-kinase. The most recent addition to this expanding family of signal transducers is the product of the human *AT* gene, which is mutated in the autosomal recessive disorder ataxia telangiectasia (87). Mutations in AT result in neurologic, immunologic, and cell-cycle abnormalities, as well as radiation-sensitivity and a predisposition to cancer. These findings hint that members

of the family of PI 3-kinase-like enzymes, including mTOR, are involved in a broad range of physiologic processes linked to control of the cell-cycle.

The growth-arrest state induced by RAP in T lymphocytes and other hematopoietic cells suggests that this protein executes a biochemical function(s) required for progression from G_1- to S-phase. In spite of the sequence homology to PI 3-kinase, mTOR possesses no detectable kinase activity toward phosphoinositides or other lipid substrates (83). However, recombinant mTOR phosphorylates itself on serine residues (83), suggesting that mTOR, ATM, and related proteins may constitute a novel family of protein serine-threonine kinases. Although the proximate substrate(s) for mTOR remains obscure, the connection to components of the machinery that control translation of mRNA is becoming increasingly compelling. The next few years should see some exciting advances in our understanding of the functions of mTOR and other members of this protein family. It is anticipated that these advances will seed the development of novel strategies for immunosuppression, and for the treatment of leukemias, lymphomas, and other cancers.

NOTE ADDED IN PROOF

While this review was being prepared for publication, Brown et al (135) reported findings which strongly support the model that mTOR represents the RAP-sensitive regulator of $p70^{S6K}$ in mammalian cells. Both the putative catalytic domain at the carboxy terminus and the amino terminal domain of mTOR were required for the regulatory action of this protein on $p70^{S6K}$ activity in vivo. Furthermore, this study shows that recombinant mTOR autophosphorylates in vitro and that this autokinase activity is sensitive to inhibition by the FKBP12 · RAP complex.

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